Journal of Forensic and Legal Medicine 16 (2009) 148–151

Contents lists available at ScienceDirect

Journal of Forensic and Legal Medicine

journal homepage: www.elsevier.com/jflm

Original Communication

What constitutes a normal ante-mortem urine GHB concentration?

Francesco Mari (Study Design and Supervision) [a,*], Lucia Politi PhD (Data Elaboration and Manuscript Writing) [a], Claudia Trignano MD [b], Maria Grazia Di Milia MSc [a], Marianna Di Padua MD (Development and Performance of Analytical Procedures) [a], Elisabetta Bertol (Study Design and Supervision) [a]

[a] Forensic Toxicology Division, Department of Anatomy, Histology and Legal Medicine, University of Florence, Italy
[b] Institute of Legal Medicine, University of Sassari, Italy

ARTICLE INFO

Article history:
Received 25 March 2008
Received in revised form 30 July 2008
Accepted 16 August 2008
Available online 29 October 2008

Keywords:
GHB
Urine
Cut-off

ABSTRACT

Gamma-hydroxybutyric acid (GHB) is endogenously produced within the central nervous system, however it is also used as a medication for the treatment of a variety of clinical conditions, sold under the name Zyrem™ in the United States and Alcover® in Europe. It is a very dangerous drug with a very limited safety margin, and is classified as a controlled substance in many countries. The interpretation of post-mortem studies of GHB concentrations is problematic; GHB can be detected in urine and blood from non-GHB users, both before and after death, and concentrations in both matrices may rise with prolonged storage. Because it is produced as a post-mortem artifact, forensically defensible cut-offs for post-mortem blood concentrations have yet to be established. Given the enormous degree of inter and intra-individual variation in GHB production that has been documented, it is unlikely they ever will. The important issue for forensic scientists is whether the detection of GHB in urine, in concentrations above some yet to be determined value, can be used as evidence for drug facilitated assault. In an attempt to see if a cut-off level could be determined we analyzed urine from 39 alcoholics who were being treated with known oral doses of Alcover® (group 1), and compared the results with concentrations found in the urine of 30 volunteers who had no exogenous GHB intake (group 2), and 30 urine specimens taken from the alcoholics before they initiated GHB therapy (Alcover® treatment group 3). More than one third (36.6%) of subjects being treated with GHB were found to have urinary GHB concentration that fell between 2.75 and 10 μg/mL. The data suggests that caution must be used when applying the currently used cut-off of 10 μg/mL.

1. Introduction

Gamma-hydroxybutyric acid (GHB) is a short-chain fatty acid, a metabolite and analogue of gamma-aminobutyric acid (GABA). It was first synthesized in 1960 for use as an intravenous anesthetic agent.[1] It is a strong central nervous system depressant because of its interaction with gamma-aminobutyric acid ($GABA_b$) receptor in addition to its reaction with a specific GHB-receptor.[2–4] Furthermore, GHB alters dopamine transmission.[5,6] It has been used as a natural product for the treatment of weight loss, and also to induce mood enhancement, treat insomnia, prevent ageing, reduce anxiety and depression, treat balding, and even as an aphrodisiac. However, GHB may produce many negative symptoms including confusion, dizziness, vomiting, nausea, bradycardia, respiratory depression, amnesia, strong sedation, and even death.[7,8] All of these symptoms have an extremely rapid onset, making it an ideal candidate drug for drug-facilitated sexual assault,[9] though the half-life of GHB is so short that such allegations may be difficult to prove.

GHB is classified a Schedule I controlled substance in the United States. However, in 2002, the US Food and Drug Administration (FDA) approved GHB for clinical use in treatment of narcolepsy (for this reason GHB is also listed in Schedule III for limited uses, and is sold under the trademark Xyrem®). In Italy GHB and its precursor, gamma-butyrolactone (GBL), have been scheduled as a controlled substance since 1999. FDA considers GBL to be an unapproved drug. In Italy the only permitted indication for GHB is the treatment of alcohol withdrawal and dependence (Alcover®). The detection and identification of GHB depends on the timeliness of sample collection. As noted, GHB is naturally present in the human body as GABA metabolite, with both ante-mortem and post-mortem specimens often containing measurable amounts of endogenous GHB. The purpose of this study was to characterize endogenous GHB concentrations found in ante-mortem urine specimens, and to demonstrate that a threat of false negatives exist when using the widely accepted cut-off of 10 μg/mL.

* Corresponding author. Tel./fax: +39 0557947208.
E-mail address: francesco.mari@unifi.it (F. Mari).

1752-928X/$ - see front matter © 2008 Elsevier Ltd and Faculty of Forensic and Legal Medicine. All rights reserved.
doi:10.1016/j.jflm.2008.08.014

2. Materials and methods

2.1. Reagents

Alcover® (17.5% GHB sodium salt) was acquired from CT Laboratorio Farmaceutico (Sanremo, Italy), nalorphine, the internal standard, from Sigma (St. Louis, MO). Hepes Buffer and *N,O*-bis-(trimetylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) were purchased from Sigma (St. Louis, MO). All other reagents were purchased from J.T.Baker (Deventer, Holland). Blanks, calibrators and controls were prepared in certified drug-free urine purchased from Polymed (Firenze, Italy).

2.2. Experimental design

All participants (n = 90, 70 males, 20 females, 20–37 years old) provided informed consent. The study population tested consisted of: 30 selected alcoholics who were being treated with known oral doses of Alcover® (20–40 mg/kg/day) (group 1); 30 volunteers who absolutely denied any exogenous GHB intake (group 2); and 30 alcohol dependent subjects who had not yet initiated therapy (20–40 mg/kg/day) with Alcover® (group 3). Specimens were donated anonymously and the subjects were not paid for their participation.

2.3. Urine collection and storage

Group 1 specimens were collected up to a maximum of 12 h after ingestion of Alcover®. They were frozen immediately at −20 °C until analysis. No preservatives were added. The maximum storage time before analysis was 5 days. This cut-off was used with the hope of minimizing the influence of in vitro GHB production. Previous studies have shown that spontaneous production of GHB does not occur within 5 days when samples were stored at −20 °C.[10]

2.4. Extraction procedure

The extraction procedure and GC-MS analysis were performed according to the method recommended by the kit maker, Biochemical Diagnostics Inc. The specimens were purified by solid phase extraction (SPE) using MultiPrep®Anion Exchange GVSA-200 Gravity Flow Column. Analysis was performed without conversion of GHB to its lactone form (GBL).[11] Aliquots of 50 μL of specimens and calibrators were spiked with 100 μL of the 0.1 mg/mL solution of nalorphine. The specimens were treated with 1 mL 0.025 M Hepes buffer pH 8.2. Each SPE column was conditioned with 3 mL of each of the following solutions: 0.2 M acetic acid in acetone, 10% HCl in H_20, and 6 mL deionized water. Each specimen was then added to the column and allowed to flow by gravity. As a wash solution, 6 mL of deionized water were added to remove excess Hepes Buffer and 2 mL of acetone to remove non-ionic contaminants. Two milliliters of 0.05 M acetic acid in methanol were used as elution solvent. The extracts were evaporated to dryness under a nitrogen stream at room temperature and derivatized with BSTFA with 1% TMCS and analyzed by GC-MS. The injection volume was 1 μL.

2.5. Instrumental analysis

Extracts were analyzed by a Thermoquest Trace GC/Finningan Polaris Q MS, equipped with a 12 m × 0.2 mm I.D. phenylmethylsilicone 5% (5 MS) capillary column with a 0.33 μm film thickness. The flow carrier gas (helium) was kept constant at 1.2 mL/min. The injector was set at 300 °C and splitless injection was employed utilizing a split valve off-time of 3 min. The column oven temperature was programmed to rise from an initial temperature of 60 °C hold for 0.5 min, increase to 130 °C at 10 °C/min, then to 300 °C at 40 °C/min, and hold at 300 °C for 3 min. The instrument was used in the full scan electron ionization mode, scanning m/z in the range 50–500 and in the acquisition selected ion monitoring (SIM) mode for GHB derivative (233, 234, 235) and for nalorphine derivative (455, 414, 440).

2.6. Quantitative analysis of GHB and validation

Quantification was performed by construction of two 5-point calibration curves that were prepared by spiking drug-free urine at the following final concentrations: 0.25, 0.5, 1.0, 2.5, 5.0 μg/mL to analyze specimens from group 2 and 3 (curve y_a), and 11.0, 22.0, 44.0, 88.0, 176.0 μg/mL to analyze specimens of group 1 (curve y_b). Blanks and positive controls (0.25, 1.8, and 3.0 μg/mL determined on curve y_a and 11.0, 25.0, and 100.0 μg/mL determined on curve y_b) were analyzed within each batch run. Inter-day precision and accuracy were calculated as relative standard deviation and bias on positive control specimens ran on five different days.

3. Results and discussion

Acceptable linear regression was obtained for both calibration curves ($y_a = 0.221x + 0.50$, $R^2 = 0.9541$; $y_b = 0.228x + 4.30$, $R^2 = 0.9969$). Inter-day precision and accuracy were always better than 15% (Table 1). The lower limit of quantification (LLOQ) was determined to be 0.25 μg/mL. Precision and accuracy data support the use of a cold compound (nalorphine in this case) as internal standard, that is evidently a much cheaper alternative to the deuterated compound.

Results obtained from individual urine specimens are summarized in Table 2, including the mean, standard deviation, and median for each group. Subjects treated with Alcover® (group 1) had GHB urine concentrations ranging from 2.75 to 91.73 μg/mL, with a median concentration of 22.9 μg/mL and a mean of 30.9 μg/mL (standard deviation 27.86). GHB-free subjects belonging to group 2 (volunteers) showed endogenous GHB levels ranging from 0.43 to 1.45 μg/mL, with a median concentration of 0.7 μg/mL and a mean of 0.8 μg/mL (standard deviation 0.26). Alcoholics with no GHB exogenous intake (group 3) were found to fall in a range between 0.43 and 1.40 μg/mL, with both mean and median at 0.9 μg/mL (standard deviation 0.30). Urine concentrations of the subjects being treated with Alcover® showed substantial inter-individual variability. So many factors, such as GHB dose, renal clearance, variations in metabolism, and different collection time after GHB intake, may affect the final measured GHB concentration, which makes it extremely difficult to draw comparisons with previously published studies where GHB has been used either as an anesthetic[12] or to treat alcoholism.[13]

Results from subjects who were not taking any exogenous GHB (groups 2 and 3), agreed with those reported in other studies. In 2002, Elian et al.[14] analyzed 670 urine specimens from individuals who were known not to be receiving GHB. Values for the entire group fell in the range of 0.34-5.75 μg/mL; in 2003 Elliot et al.[15]

Table 1
Validation data: average concentration, accuracy and inter-day precision (n = 5)

	Curve y_a			Curve y_b		
Nominal concentration (μg/mL)	0.25	1.8	3	11	25	100
Average concentration (μg/mL)	0.27	1.54	2.91	10.19	21.42	85.31
Accuracy (%)	8.64	3.29	2.59	7.35	14.32	14.69
Inter-day precision (%)	−6.79	14.40	3.03	5.21	2.45	3.93

Table 2
GHB concentrations of the urine samples from group 1 (alcoholics in treatment with 20–40 mg/kg/day of Alcover®), group 2 (volunteers with no GHB exogenous intake), and group 3 (alcoholics with no GHB exogenous intake)

Sample no.	GHB concentration (μg/mL)		
	Group 1	Group 2	Group 3
1	7.4	0.7	0.7
2	13.2	0.5	0.8
3	91.7	0.6	0.7
4	6.6	0.8	0.6
5	23.6	0.8	1.0
6	67.1	1.1	0.9
7	6.7	0.5	1.4
8	55.5	0.4	1.4
9	26.9	0.6	1.0
10	59.7	0.7	0.8
11	57.5	0.5	0.6
12	74.4	1.0	1.3
13	35.8	1.5	0.4
14	4.0	1.4	0.5
15	2.8	0.7	0.9
16	2.9	0.9	1.0
17	3.6	0.6	1.0
18	63.6	0.8	0.5
19	40.3	0.9	0.7
20	7.8	1.0	1.0
21	89.6	0.7	1.2
22	22.1	0.7	1.3
23	7.1	0.7	0.6
24	27.0	0.8	1.0
25	55.5	1.0	0.8
26	34.5	0.6	1.4
27	2.9	1.2	0.8
28	5.7	0.7	1.0
29	12.6	0.9	1.3
30	20.2	0.9	1.4
Mean	30.9	0.8	0.9
Standard deviation	27.9	0.3	0.3
Median	22.9	0.7	0.9

Table 3
GHB concentration in non-users observed in prior studies of ante-mortem urine

Author (reference)	Year	Number	Type donor	Observed maximal conc.
Abades[25]	2007	5	GHB-free volunteers	0.21 ± 0.14 μg/ml
LeBeau[21]	2006	207	GHB-free volunteers	0–2.70 μg/mL
Moriya[22]	2006	20	GHB-free volunteers	Less than 1 μg/ml
Berankova[23]	2006	1	GHB-free volunteer	None detected
Shima[24]	2005	30	GHB-free volunteers	0.22–1.42 μg/ml
Crookes[17]	2004	50	GHB-free volunteers	1.36 μg/mL max
Yeatman[16]	2003	50	GHB-free volunteers	3.5 μg/ml
Elliott[15]	2001	119	GHB-free volunteers	<3 μg/ml in every case
Elian[14]	2002	240	GHB-free-volunteers	4.1 μgml
Kerrigan[10]	2002	*n* = ?	(Pooled drug free ante-mortem)	0 First 6 days

analyzed 144 specimens from drug free subjects; the maximal urinary GHB concentration he found was 3 μg/mL. Also in 2003 Yeatman and Reid,[16] measured urinary endogenous levels of GHB urine specimens voluntarily provided by 55 subjects. The concentrations measured ranged from 0.9 to 3.5 μg/mL. In 2004 Crookes et al.[17] quantified endogenous GHB concentrations in 50 urine specimens from women and found that concentrations ranged from 0.1 μg/mL to 1.46 μg/mL; they proposed to separate endogenous GHB production from exogenous administration by using a 5 μg/mL cut-off for urine GHB concentration. Many authors, Kavanagh et al.,[18] McCusker et al.,[19] Yeatman and Reid[16] and Eklund et al.,[20] have experimented with increasing higher cut-offs (2 μg/mL, 5 μg/mL, 10 μg/mL and 20 μg/mL, respectively) whose diagnostic accuracy remains debatable. Recently, in 2006, Le Beau et al.[21] suggested reinstating a 10 μg/mL for urinary endogenous GHB cut-off. His suggestion was based on the results he obtained when he analyzed 207 urinary specimens and found urinary GHB concentrations ranging from 0.00 to 2.70 μg/mL. The 10 μg/mL cut-off is now widely accepted. However, if we apply the 10 μg/mL cut-off to the subjects known to be taking Alcover® examined in this study (group 1, Table 2), 36.6% will be false negatives, with GHB concentrations ranging from 2.75 to 7.80 μg/mL. Based upon our results it would appear that using a 10 μg/mL cut-off risks underestimating GHB use/abuse since it cannot discriminate endogenous from exogenous production. Our conclusion is not novel. As illustrated in Table 3. Therefore, we suggest that a urinary GHB cut-off value between 3 and 10 μg/mL should be considered as clue of exogenous administration.

Unfortunately, neither immunoassays nor other rapid methods of diagnosis currently exist. We have described here a useful method that can be used as screening, confirmation and quantification of GHB. Lowering the cut-off may go a long way toward allowing the documentation of previously undiagnosed instances of GHB administration.

Conflict of interest statement

None.

Funding

None.

Ethical Approval

Ethical approval was obtained from local Ethics Committee.

References

1. Lborit H. Sodium 4-hydroxybutyrate. *Int J Neuropharmacol* 1964;**3**:433–52.
2. Tunnicliff G. Sites of action of gamma-hydroxybutyrate (GHB)-a neuroactive drug with abuse potential. *Clin Toxicol* 1977;**35**:581–90.
3. Snead III OC. Evidence for a G protein-coupled gamma-hydroxybutyric acid receptor. *J Neurochem* 2000;**75**:1986.
4. Colombo G, Agabio R, Lobina C, Reali R, Gessa GL. Involvement of GABA(A) and GABA(B) receptors in the mediation of discriminative stimulus effects of gamma hydroxybutyric acid. *Physiol Behav* 1998;**64**:293–302.
5. Feigenbaum JJ, Howard SG. Does gamma-hydroxybutyrate inhibit or stimulate central dopamine release? *Int J Neurosci* 1996;**88**:53–69.
6. Diana M, Mereu G, Mura A, Fadda F, Passino N, Gessa GL. Low doses of γ-hydroxybutyric acid stimulate the firing of dopaminergic neurons in unanesthetized rats. *Brain Res* 1991;**566**:208.
7. Couper FJ, Marinetti LJ. Γ-hydroxybutyrate (GHB)-effects on humans performance and behaviour. *Forensic Sci Rev* 2002;**14**:101–21.
8. Li J, Stokes SA, Woeckener A. A tale of novel intoxication: a review of the effects of gamma-hydroxybutyric acid with recommendations for management. *Ann Emerg Med* 1998;**31**:729–36.
9. Stillwell ME. Drug-facilitated sexual assault involving gamma-hydroxybutyric acid. *J Forensic Sci* 2002;**47**:1133–4.
10. Kerrigan S. In vitro production of gamma-hydroxybutyrate in antemortem urine samples. *J Anal Toxicol* 2002;**26**:571–4.
11. Multi-prep® "no vacuum" gravity series GVSA-200 method for the measurement of gamma-hydroxybutyrate (GHB) in serum and urine using GC/MS. Biochemical Diagnostics, Inc.; May 2003.
12. Pöschl J, Kölker S, Bast T, Brüssau J, Ruef P, Linderkamp O, et al. Gamma-hydroxybutyric acid sedation in neonates and children undergoing MR imaging. *Klin Padiatr* 2007;**219**:217–9.
13. Haller C, Thai D, Jacob III P, Dyer JE. GHB urine concentrations after single-dose administration in humans. *J Anal Toxicol* 2006;**30**:360–4.
14. Elian AA. Determination of endogenous gamma-hydroxybutyric acid (GHB) levels in antemortem urine and blood. *Forensic Sci Int* 2002;**128**:120–2.
15. Elliott SP. The presence of gamma-hydroxybutyric acid (GHB) in post-mortem biological fluids. *J Anal Toxicol* 2001;**25**:152.
16. Yeatman DT, Reid K. A study of urinary endogenous gamma-hydroxy-butyrate (GHB) levels. *J Anal Toxicol* 2003;**27**:40–2.
17. Crookes CE, Faulds MC, Forrest ARW, Galloway JH. A reference range for endogenous gamma-hydroxybutyrate in urine by GC/MS. *J Anal Toxicol* 2004;**28**:644–9.

18. Kavanagh PV, Kenny P, Feely J. The urinary excretion of gamma-hydroxybutyric acid in man. *J Pharm Pharmacol* 2001;**53**:399–402.
19. McCusker RR, Paget-Wilkes H, Chronister CW, Goldberger BA. Analysis of gamma-hydroxybutyrate (GHB) in urine by gas-chromatography-mass spectrometry. *J Anal Toxicol* 1999;**23**:301–5.
20. Eklund A, Eriksson M. Gamma-hydroxybutyrate (GHB): endogenous concentrations, abuse, traffic accidents and death. In: Presented at the 38th meeting of the international association of forensic toxicologists, Helsinki, Finland; 2000.
21. Le Beau MA, Montgomery MA, Morris-Kukoski C, Schaff JE, Deakin A, Levine B. A comprehensive study on the variations in urinary concentrations of endogenous gamma-hydroxybutyrate (GHB). *J Anal Toxicol* 2006;**30**:98–105.
22. Moriya F, Nishimura H, Furumiya J, Hashimoto Y. Effects of drinking and smoking on endogenous levels of urinary gamma-hydroxybutyric acid, a preliminary study. *Leg Med Tokyo* 2006;**8**:231–4.
23. Beránková K, Mutnanská K, Balíková M. Gamma-hydroxybutyric acid stability and formation in blood and urine. *Forensic Sci Int* 2006;**161**:158–62.
24. Shima N, Miki A, Kamata T, Katagi M, Tsuchihashi H. Urinary endogenous concentrations of GHB and its isomers in healthy humans and diabetics. *Forensic Sci Int* 2005;**149**:171–9.
25. Abanades S, Farré M, Segura M, Pichini S, Pastor A, Pacifici R, et al. Disposition of gamma-hydroxybutyric acid in conventional and nonconventional biologic fluids after single drug administration: issues in methodology and drug monitoring. *Ther Drug Monit* 2007;**29**:64–70.